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Diallyl Disulfide Increases CDKN1A Promoter-Associated Histone Acetylation in Human Colon Tumor Cell Lines

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Diallyl disulfide (DADS) is an organosulfur compound from garlic, which inhibits colon tumor cell proliferation. In a previous study, we have shown that in Caco-2 and HT-29 cells DADS (200 μM) increases global histone acetylation, CDKN1A mRNA, and p21^{waf1} protein levels and induces G_2/M cell cycle arrest. These results suggested that DADS could inhibit cell proliferation through at least in part a transcriptional activation of CDKN1A expression involving histone acetylation. In this study, using chromatin immunoprecipitation assays, we demonstrate that in Caco-2 and HT-29 cells histone H4 and/or H3 acetylation is increased within CDKN1A promoter after 3 and 6 h treatments with DADS. These results strongly suggest that histone acetylation, a molecular mechanism implicated in the regulation of gene expression, could account for the induction of CDKN1A expression and the antiproliferating effects of DADS in colon tumor cells.

KEYWORDS: Diallyl disulfide; histone acetylation; human colon tumor cells; chromatin immunoprecipitation; CDKN1A gene

INTRODUCTION

The protein p21^{waf1} is involved in the regulation of diverse biological processes such as cell cycle progression, DNA replication and repair, transcription, and apoptosis (*1*). The p21^{waf1} protein is a negative regulator of cell cycle progression, which has been shown to mediate G_1/S and G_2/M arrests. Its implication in the control of checkpoint transitions relies mainly on its ability to inhibit cyclin/cyclin-dependent kinase complexes (*2*). The p21^{waf1} protein is encoded by the CDKN1A gene whose expression is mainly controlled at the transcriptional level by both p53-dependent and p53-independent mechanisms. CDKN1A transcription can be induced through two p53 binding sites located within its promoter and influenced by epigenetic mechanisms such as histone acetylation (*3*). Interactions between transcriptional activators and histone acetylases as well as remodeling complexes are thought to facilitate the loading of the initiation complex. Acetylation of specific residues, which occurs within the N-terminal domain of core histones H2A, H2B, H3, and H4, is one of the mechanisms involved in gene expression activation through an increase in DNA accessibility to transcription factors (*4*).

In colon tumor cell lines, well-known histone deacetylase inhibitors (HDACi) like TSA and butyrate have been shown to inhibit cell proliferation. In HCT116 and HT-29 cells, the inhibition of proliferation induced by these compounds was correlated with an increase in CDKN1A expression and in histone acetylation (*5*−*7*). The transcriptional activation of CDKN1A is thought to result from hyperacetylation of the promoter-associated histones. In chromatin immunoprecipitation (ChIP) experiments, an increase in histones H3 and H4 acetylation within CDKN1A promoter is induced by both TSA and butyrate in Colo-320 and SW116 cells or by butyrate in HT-29 cells (*8*−*10*).

Other molecules known to modulate colon tumor cell proliferation do have the ability to modify histone acetylation. Indeed, we have shown that diallyl disulfide (DADS), a sulfur compound from garlic, induces G_2/M cell cycle arrest in two human colon tumor cell lines (HT-29 and Caco-2) (*11*, *12*). In both cell lines, DADS antiproliferative effects were associated with increases in CDKN1A mRNA and p21^{waf1} protein levels and transient changes in histone acetylation, suggesting that DADS transcriptionnally activates CDKN1A expression through a modification of histone acetylation within its promoter (*12*). Using western blotting, we observed an increase in the acetylation of both histones H4 and H3 in Caco-2 cells, whereas in HT-29 cells no modification of histone H4 acetylation was noticed (**Table 1**). The aim of the present study was to investigate whether the hyperacetylation induced by DADS concerned the histones H4 and H3 associated with the proximal region of the CDKN1A promoter in Caco-2 and HT-29 cells. Thus, we performed ChIP assays with antibodies directed against acetyl-H4 or acetyl-H3.

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Table 1. Summary of DADS Effects on Histone H4 and Histone H3 Acetylation in Caco-2 and HT-29 Cells[a]

	incubation time (h)	Western blotting[b] acetyl-H4	Western blotting[b] acetyl-H3 K14	ChIP[c] acetyl-H4	ChIP[c] acetyl-H3
Caco-2 cells	3	↑	↑	↑	↑
	6	↑	↑	↑	↑
	24	↔	↔	ND	ND
HT-29 cells	3	↔	↔	↔	↑
	6	↔	↑	↔	↑
	24	↔	↔	ND	ND

[a] For all experiments, cells were seeded at a density of 0.2×10^5/100 mm Petri dish. Three days later, cells were treated with DMSO or 200 μM DADS for 3, 6, or 24 h. ↔, no significant modification; ↑, significant increase; ND, not determined. [b] Summary of results previously described (*12*). Analysis of histone H3 K14 and H4 acetylation states was performed by Western blotting using antibodies directed against acetyl-H4 and acetyl-H3 K14. [c] Summary of results of the present study. Analysis of histone H3 and H4 acetylation within a proximal region of CDKN1A promoter was performed by ChIP using antibodies directed against acetyl-H4 and acetyl-H3 and a set of primers amplifying the region –575/–321 bp (from the start site) of CDKN1A promoter as described in the Materials and Methods.

MATERIALS AND METHODS

Reagents. Dimethyl sulfoxide (DMSO), DADS (80% purity, the remaining 20% being diallyl trisulfide and diallyl sulfide), TSA, sodium butyrate, salmon testes DNA, bovine serum albumin (BSA), and proteinase K were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). All reagents for cell culture were from Invitrogen (Cergy Pontoise, France) excepted for DMEM (BioWhittaker, Emerainville, France) and amino acids (Biomedia, Boussens, France). Protease inhibitor cocktail was from Roche Diagnostics (GmbH, Mannheim, Germany), and protein A/G plus-agarose was from Santa-Cruz Biotechnology (Santa-Cruz, CA).

Cell Culture. Two human colon adenocarcinoma cell lines were used, parental Caco-2 and HT-29 $Glc^{-/+}$ cells. Both cell lines were cultivated under 10% CO_2, at 37 °C, in DMEM (with 4.5 g/L glucose), supplemented with 10% (HT-29) or 20% (Caco-2) heat-inactivated fetal calf serum, penicillin (10 units/mL)/streptomycin (10 units/mL), and 4 mM glutamine. Non-essential amino acids (1%) were added to Caco-2 media. Media completed with DMSO, DADS, butyrate, and TSA were prepared to reach final concentrations of 0.1% DMSO, 200 μM DADS, 5 mM butyrate, and 1 μM TSA as previously described (*12*). For all experiments, cells were seeded at low density, 0.2×10^6 cells in 100 mm Petri dishes. Four days after seeding, cells were submitted to the different treatments for 0, 3, or 6 h, incubation times favorable to observe an effect of DADS on histone acetylation (**Table 1**).

ChIP. Following DMSO, DADS, butyrate, or TSA treatments, Caco-2 and HT-29 cells were treated with 1% formaldehyde for 10 min at room temperature, then washed twice with ice-cold phosphate-buffered saline (PBS), scraped, and collected by centrifugation (700*g*, 5 min at 4 °C). Cells were then sonicated in 500 μL of 50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, 1 mM PMSF, protease inhibitor cocktail for five cycles of 10 s, to an average length of 300 base pairs (Branson Sonifier Cell Disruptor B15, Paris, France), and debris was removed from samples by centrifugation (8000*g*, 10 min). Supernatants were analyzed by spectrophotometer at 260 nm and diluted in RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM PMSF and protease inhibitor cocktail to obtain, for each sample, the equivalent of 2×10^6 or 6×10^6 cells, for Caco-2 and HT-29 cells, respectively. The chromatin solution was precleared with 110 μL of salmon testes DNA/BSA/protein A/G plus-agarose (5 μg/5 μg/100 μL), rocking for 30 min at 4 °C. Following centrifugation (14000*g*, 2 min, 4 °C), chromatin was incubated with 10 μg of anti-acetylated histone H4 or 5 μg of anti-acetylated histone H3 (Upstate Biotechnology) overnight at 4 °C with agitation. Antibody−protein−DNA complexes were incubated (2 h, 4 °C with agitation) with 110 μL of the salmon testes DNA/BSA/protein A/G plus-agarose solution. Complexes were washed as previously described (*13*) and extracted twice with 250 μL of 1% SDS/0.1 M $NaHCO_3$. Eluates were incubated with 0.1 mM NaCl at 68 °C overnight and then digested with 2 μL of proteinase K (20 mg/mL), 20 μL of EDTA 0.25 M, and 40 μL of 0.5 M Tris-HCl, pH 6.8 (1 h at 55 °C). DNA samples were then extracted with 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1) followed by precipitations with ethanol. Pellets were resuspended in 20 μL of water and submitted to polymerase chain reaction (PCR).

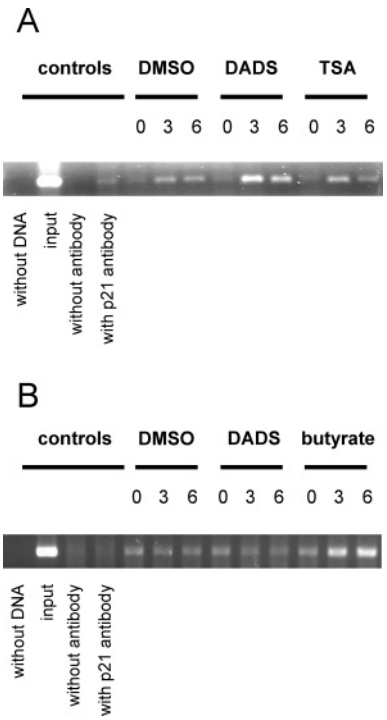

Figure 1. DADS effects on the acetylation of histones H4 associated with the CDKN1A promoter in Caco-2 (**A**) and HT-29 (**B**) cells. Cells were treated with DMSO, 200 μM DADS, 1 μM TSA, or 5 mM butyrate for 0, 3, or 6 h. Chromatin was immunoprecipitated with antibodies directed against acetylated H4 histones. Extracted DNA was amplified using a set of primers as described in the Materials and Methods. Images are representative of two independent experiments.

PCR Analysis of Immunoprecipitated DNA. PCR amplification was performed with 2 μL (input) or 4 μL (immunoprecipitated DNA) of DNA solution supplemented with 5 μL of 10× buffer 2 mM $MgCl_2$, 1 μL of dNTP mix (10 mM), 0.5 μL of each 100 nM solution of primers, 0.5 μL of *Taq* polymerase hotstart (Eppendorf, Le Pecq, France), and water to a final volume of 50 μL. A set of primers amplifying −575 to −321 bp from the transcription start site was used as follows: 5′-GGT GTC TAG GTG CTC CAG GT-3′ and 5′-GCA CTC TCC AGG AGG ACA CA-3′ (*14*). The PCR cycle consisted of 15 min at 95 °C, 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and 10 min at 72 °C for 35 cycles. PCR products were separated on a 2% agarose gel, visualized with ethidium bromide using a fla-3000 phosphoimager (Fujifilm, Paris, France).

RESULTS

We explored the DADS effect on the acetylation of CDKN1A promoter-associated histones in Caco-2 and HT-29 cells, after 3 and 6 h treatments. The histone H4 and H3 acetylation level within CDKN1A promoter was studied by ChIP, using anti-acetylated histones H4 and H3 antibodies and a set of primers, targeting a region of the promoter (−575/−321 bp).

To our knowledge, no data were available in Caco-2 cells concerning the effect of HDACi on the acetylation of CDKN1A promoter-associated histones. We observed in our previous study that 1 μM TSA and 5 mM butyrate induced histone H4

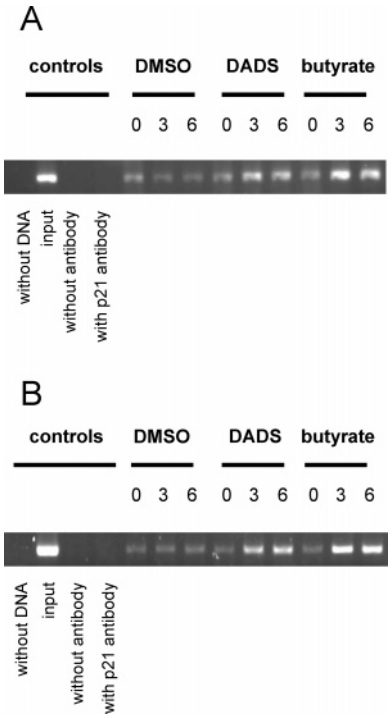

Figure 2. DADS effects on the acetylation of histones H3 associated with the CDKN1A promoter in Caco-2 (**A**) and HT-29 (**B**) cells. Cells were treated with DMSO, 200 μM DADS, or 5 mM butyrate for 0, 3, or 6 h. Chromatin was immunoprecipitated with antibodies directed against acetylated H3 histones. Extracted DNA was amplified using a set of primers as described in the Materials and Methods. Images are representative of two independent experiments.

hyperacetylation in Caco-2 cells; we thus determined whether they could be considered as positive controls for our ChIP experiments. TSA and butyrate did increase the acetylation of histone H4 and H3 within the targeted region of the CDKN1A promoter, after 3 and 6 h treatments as illustrated in **Figures 1A** and **2A**.

As shown in **Figure 1A**, in Caco-2 cells, after 3 and 6 h of treatment, 200 μM DADS increased the acetylation level of histone H4 associated to CDKN1A promoter as compared to DMSO controls. In HT-29 cells, butyrate was used as a positive control referring to a previous study (*8*). After 3 and 6 h, as expected, butyrate enhanced CDKN1A promoter-associated histone H4 acetylation whereas the level of acetylated histones H4 within CDKN1A promoter was not modified in response to 200 μM DADS (**Figure 1B**). As shown in **Figure 2A**,**B**, 200 μM DADS also enhanced the acetylation of histones H3 associated with CDKN1A promoter after 3 and 6 h of treatments in both cell lines.

DISCUSSION

DADS inhibitory effects on chemically induced carcinogenesis in various organs including colon have been demonstrated in rodent models (*15*, *16*). Further studies have explored the mechanisms, which may explain these anticarcinogenic properties. DADS anti-initiating and antipromoting effects could partly result from its ability to increase drug metabolizing−enzyme activity and to inhibit the proliferation of tumor cells, respectively (*16*, *17*). Recently, increasing interest has been focused on DADS ability to modulate gene expression (*18*, *19*). In tumor colon cells, modulations of the expression of a large subset of genes, involving alterations in ERK pathway signalling, were associated to DADS antiproliferating effects (*19*).

Previous data showed that DADS could enhance global histone acetylation in vivo (*20*) and in vitro (*12*, *21*). The present study reports for the first time the ability of DADS to increase the acetylation of histones associated to a gene promoter. Indeed, in Caco-2 and HT-29 cells, DADS increases CDKN1A promoter-associated histone acetylation.

Our results show DADS ability to increase the acetylation of histones associated to the −575/−321 region of the CDKN1A promoter. Moreover, in Caco-2 cells treated with DADS for 6 h, the acetylation of histones H4 associated to the −254/+31 promoter region was also enhanced (data not shown). In colon tumor cell lines, butyrate and TSA were shown to induce acetylation of CDKN1A promoter-associated histones in regions including TATA box and/or Sp1 sites as well as in upstream regions of the promoter (*8*−*10*). Interestingly, it has been reported recently that histone hyperacetylation could occur in various regions between −3841/+7438 bp of the gene (*22*). Thus, it would be interesting to further extend the analysis of DADS effect to downstream promoter regions as well as nonpromoter regions.

In response to DADS, histone H4 acetylation status within CDKN1A promoter was increased rapidly after 3 and 6 h of

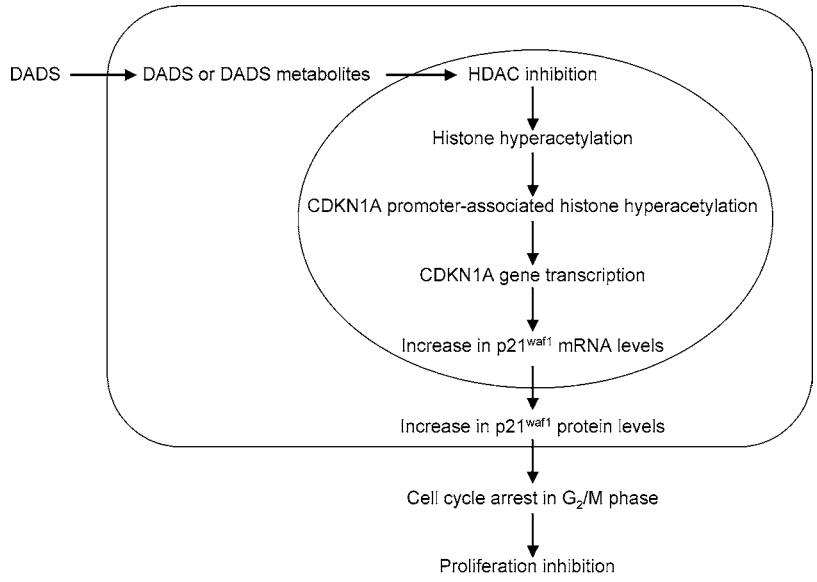

Figure 3. Schematic model for DADS-induced inhibition of the proliferation of HT-29 and Caco-2 cells.

treatment, in Caco-2 cells, but remained unchanged in HT-29 cells. Moreover, DADS also enhanced rapidly the acetylation of histones H3 within the target promoter in both Caco-2 and HT-29 cells. These results are consistent with data obtained in our previous study, showing that (i) DADS increased the acetylation of histone H3 on the lysine 14 in both cell lines and (ii) DADS effect on histone H4 acetylation was seen only in Caco-2 cells (**Table 1**) (*12*). Because butyrate could induce hyperacetylation of promoter-associated histone H4 in this HT-29 Glc −/+ cells as well as in parental HT-29 cells (*8*), the absence of modification of histone H4 in response to DADS in our experiments might not be linked to the HT-29 subline. Nevertheless, this hypothesis cannot be excluded since DADS effect on histone H4 acetylation has never been explored in parental HT-29 cells.

As DADS induced CDKN1A promoter-associated histone H4 and/or H3 hyperacetylation in Caco-2 and HT-29 cells, it may share some mechanisms of action with other HDACi. Interestingly, it has been reported that butyrate effects on CDKN1A transcriptional activation involved the recruitment of the transcription factors ZBP89, Sp1, and a HAT (GCN5 or p300) in HT-29 cells (*8*, *23*). SAHA, another HDACi, induced a decrease of promoter-associated HDAC1, HDAC2, and c-myc and an increase of promoter-associated RNA polymerase II in ARP-1 myeloma cells (*22*). Thus, the factors involved in DADS-induced histone hyperacetylation within the target promoter deserve to be determined. Further molecular investigations are needed and might partially elucidate the differences observed between Caco-2 and HT-29 cells in response to DADS.

Recent data have suggested that other molecular mechanisms are necessary for CDKN1A transcriptional activation by HDACi (*8*, *24*). Moreover, it has been shown that in hepatic cells, the activation of the CDKN1A gene under cytokine stimulation implicated the transcription factor STAT3. At the CDKN1A promoter, STAT3 DNA binding resulted in histone H3 acetylation and in the recruitment of BRG1, a component of the SWI/SNF chromatin-remodeling complex (*25*). Therefore, it would be interesting to further analyze DADS effect on chromatin remodeling in Caco-2 and HT-29 cells.

In conclusion, our study strongly suggests the involvement of histone acetylation in DADS-induced modulation of gene expression. The increase by DADS of CDKN1A promoter-associated histone acetylation might be one of the mechanisms involved in the induction of CDKN1A expression, as well as in DADS antiproliferative effects. On the basis of previous and current findings, we propose a model for DADS-induced inhibition of the proliferation of HT-29 and Caco-2 cells (**Figure 3**). We can notice that this mechanism is shared by sulforaphane, another natural HDACi found in broccoli (*26*). As recently suggested, the efficiency of such dietary HDAC inhibitors in cancer prevention through modulation of gene expression during a lifetime exposure should not be dismissed and warrants further investigations (*27*).

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